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Interaction of Nkx3.1 and p27^{kip1} in Prostate Tumor Initiation

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The homeodomain transcription factor Nkx3.1 and the cyclin-dependent kinase inhibitor p27^{kip1} have both been implicated in prostate tumor suppression. In addition, both of these molecules demonstrate haploinsufficiency for tumor suppression, in which loss of a single allele is sufficient to lead to the development of preneoplastic or neoplastic lesions. We have generated mice carrying compound mutant alleles of *Nkx3.1* and *p27* to explore the roles of these factors in prostate tumorigenesis. Our results indicate that Nkx3.1 and p27^{kip1} cooperate to suppress the proliferation of prostatic epithelial cells and the formation of preneoplastic lesions resembling prostatic intraepithelial neoplasia. Cooperativity was most evident with complete loss of at least one of the two genes because compound heterozygous mice exhibited a prostatic phenotype that was no more severe than that of single heterozygous mutants. Thus Nkx3.1 and p27^{kip1} regulate prostatic epithelial cell proliferation and tumor initiation by affecting both haploinsufficient and nonhaploinsufficient pathways. *(Am J Pathol 2004, 164:1607–1614)*

Prostate tumor development is contingent on the accumulation of mutations in multiple oncogenes and tumor suppressor genes. Recent human and transgenic mouse model studies have established roles for two tumor suppressor genes, the homeodomain transcription factor Nkx3.1 and the cyclin-dependent kinase inhibitor p27^{kip1} in prostate tumorigenesis. The *NKX3.1* gene is localized to human chromosome 8p21, a region that undergoes loss of heterozygosity with high frequency in prostate cancers and prostatic intraepithelial neoplasia (PIN) lesions.[1,2] Evidence that *NKX3.1* is a relevant prostate tumor suppressor gene targeted by loss of heterozygosity at 8p21 has come from studies that show loss of NKX3.1 protein expression in a majority of human prostate tumors.[3,4] Loss of NKX3.1 protein expression is also significantly correlated with tumor progression.[3] Mice with deletion of one or both alleles of *Nkx3.1* develop prostatic epithelial hyperplasia and PIN[4–6] indicating that *Nkx3.1* functions as an haploinsufficient tumor suppressor gene. Recent evidence indicates that Nkx3.1 regulates the exit of regenerating prostatic epithelial cells from the cell cycle in a dosage-sensitive manner, thereby leading to hyperplasia.[7] Furthermore, in mice, loss of *Nkx3.1* cooperates with loss of the tumor suppressor, *Pten*, to promote prostate tumorigenesis.[8]

Similar to NKX3.1, expression of the cyclin-dependent kinase inhibitor p27^{KIP1} is lost in a large fraction of human prostate carcinomas, and reduced or absent expression of p27^{KIP1} correlates with poor patient prognosis.[9] Deletion of *p27* in mice also results in prostatic epithelial hyperplasia and cooperates with loss of *Pten* to promote prostate tumor development.[10,11] Finally, p27^{kip1} also demonstrates haploinsufficiency for tumor suppression because loss of one p27 allele can be sufficient for expression of the tumorigenic phenotype.[11,12]

To examine the roles of *Nkx3.1* and *p27* in regulating prostate tumorigenesis in particular with regards to haploinsufficiency, we have generated and analyzed mice carrying all possible permutations of loss-of-function alleles of the two genes. Our results indicate cooperativity between Nkx3.1 and p27^{kip1} in suppressing epithelial proliferation and development of PIN lesions. Furthermore, these effects of Nkx3.1 and p27^{kip1} are mediated via both haploinsufficient and nonhaploinsufficient pathways.

Materials and Methods

Animals and Histopathology

Nkx3.1 and *p27* mutant mice have been described previously.[5,13] Mice were genotyped by polymerase chain reaction on tail DNA. *Nkx3.1*$^{+/-}$*p27*$^{+/-}$ mice generated on a mixed C57BL6J/129Sv background were intercrossed to generate the compound mutant mice used for analyses (Table 1). Not all offspring from the matings were entered into the study. The urogenital tract, including prostate, seminal vesicles, and testes were harvested and fixed in 4% paraformaldehyde. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E)

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Table 1. Number of Mice Generated and Sacrificed by Genotype and Age

Age (weeks)	p+/+ n+/+	p+/+ n+/−	p+/+ n−/−	p+/− n+/+	p−/− n+/+	p+/− n+/−	p+/− n−/−	p−/− n+/−	p−/− n−/−	Total
12	8	6	6	8	2	15	5	4	3	57
24	15	4	4	10	7	19	11	ND	4	74
36	12	8	6	15	9	16	10	10	10	96
Total	35	18	16	33	18	50	26	14	17	227

ND, not done.

and evaluated histologically by a blinded pathologist (IAE). Prostate pathology was scored according to the following histopathological grading scale, which is a modification of the score suggested by Park and colleagues.[14] Normal, benign prostatic epithelium with normal nuclear morphology. Grade 1: Lesion demonstrating prostatic epithelial hyperplasia without atypia. Atypia here refers to the presence of nuclear crowding, nuclear elongation, and hyperchromasia. Grade 2: Lesion showing prostatic epithelial hyperplasia with mild atypia. Grade 3: Lesion showing prostatic epithelial hyperplasia with moderate atypia. Grade 4: Lesion showing prostatic epithelial hyperplasia with marked atypia.

Morphometry

Paraffin sections were stained using the Feulgen stain (Chroma Vision, San Juan Capistrano, CA). Nuclear images were extracted from the Feulgen-stained histological sections using the Cell Finder software and individual nuclei were analyzed using the Nuclear Grade software (Bacus Laboratories Inc.). Image morphometric nuclear grading was performed using the software as described.[15] Raw data of DNA mass and nuclear size were then imported into Excel spread sheet and then into SPSS statistical program. The control and the double-knockout groups were then compared using Student's *t*-test.

Western Blot Analysis

Prostate extracts were subjected to Western blot analysis as described.[16] Primary antibodies used were polyclonal anti-Nkx3.1 antibody[17] at 1:20 dilution and polyclonal anti-p27^{kip1} antibody (1:1000, Santa Cruz).

Immunohistochemistry

Immunohistochemical staining was performed using the high-temperature (microwave) antigen retrieval method as described.[17,18] The following primary antibodies were used: rabbit polyclonal anti-Nkx3.1 antibody at 1:100 dilution,[17] rabbit polyclonal anti-p27^{kip1} (1:100, Santa Cruz), rabbit polyclonal anti-Ki67 antibody (1:1000, Novocastra) and mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (1:200, Santa Cruz). Testes present on the same slide served as internal controls for Ki-67 and PCNA staining. Sections were developed using 3,3′-diaminobenzidine in Tris-buffered saline and 0.003% hydrogen peroxide, counterstained with hematoxylin, dehydrated, cleared, and mounted for viewing. For determination of the Ki-67- and PCNA-labeling indices, a minimum of 1000 cells were counted. All counts were done in the anterior lobes of the prostates for consistency.

Terminal dUTP Nick-End Labeling (TUNEL) Assay

The TiterTACS kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions for detection of apoptotic cells. Testes present on the same slide served as internal controls. A minimum of 1000 cells in the anterior lobes was counted for determination of the apoptotic index.

Results

Nkx3.1 and p27^{kip1} Cooperate to Suppress the Development of Prostatic Epithelial Hyperplasia and Dysplasia

Previous studies have shown that loss of either *Nkx3.1* or *p27* could cooperate with loss of the *Pten* tumor suppressor gene in mice to promote prostate tumorigenesis.[8,11] To determine whether loss of *Nkx3.1* and loss of *p27*

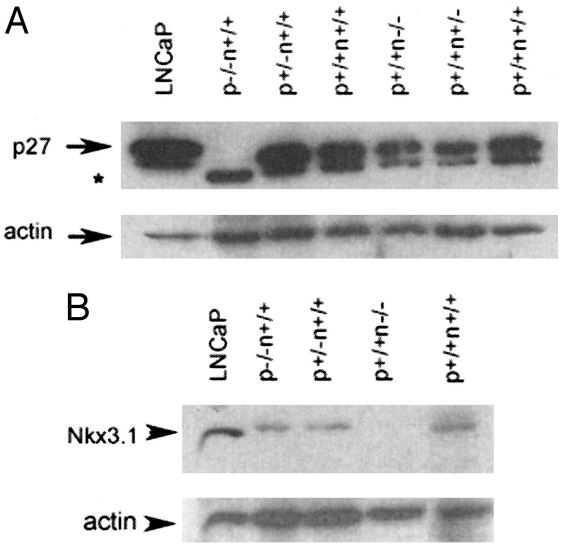

Figure 1. Expression of p27^{kip1} and Nkx3.1 in prostates of mutant mice by Western blot analyses. **A:** Expression of p27^{kip1} protein in prostates of mice of indicated genotypes. *, Nonspecific band. **B:** Expression of Nkx3.1. LNCaP human prostate cancer cell line extracts were used as controls. Actin was used as loading control.

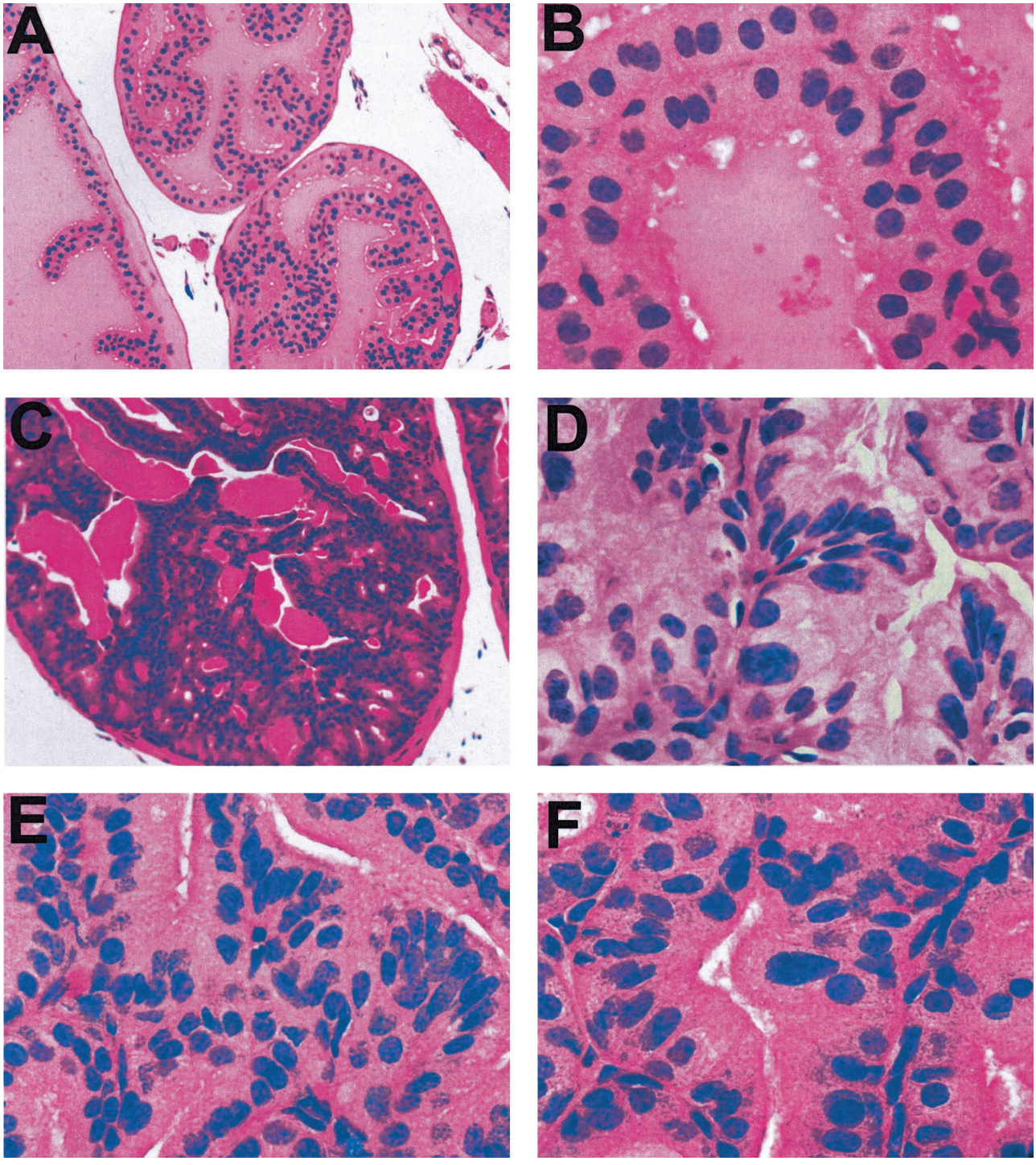

Figure 2. Prostate histopathology in *p27* × *Nkx3.1* compound mutant mice. Anterior prostates from 36-week-old mice were stained by H&E and examined histologically. **A** and **B:** Normal-looking prostate from a $p27^{+/+}Nkx3.1^{+/+}$ animal scored as grade 0. **C** to **F:** Prostatic lesions from $p27^{-/-}Nkx3/1^{-/-}$ mice showing prostatic epithelial hyperplasia with atypia including marked nuclear crowding, nuclear elongation, and hyperchromasia. Original magnifications: ×20 (**A**, **C**); ×100 (**B**, **D–F**).

cooperate in prostate tumorigenesis, we generated cohorts of compound mutant mice corresponding to the nine possible genotypes (Table 1). Using Western blot analyses, we determined that lack of Nkx3.1 does not significantly affect $p27^{kip1}$ expression levels (Figure 1A). Conversely, loss of p27 also does not significantly alter Nkx3.1 levels (Figure 1B). The prostates of mutant animals were examined for histopathological abnormalities at various ages (12 weeks, 24 weeks, and 36 weeks). As reported previously[4–6] $Nkx3.1^{-/-}$ and $Nkx3.1^{+/-}$ mutant mice display prostatic epithelial hyperplasia and dysplasia (data not shown). Lesions were identified both in the anterior and dorsolateral lobes of the prostate, but they were more extensive in the anterior lobe. For consistency,

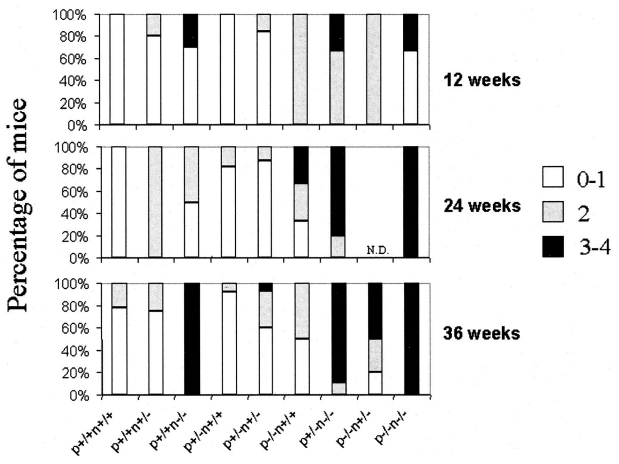

Figure 3. Analysis of histopathological scores in cohorts of 12-week-old, 24-weekold, and 36-week-old compound mutant animals generated as described in Table 1. The percentages of mice with different histological scores are indicated. N.D., not done.

all further analyses described herein were performed on the anterior prostate. $p27^{-/-}$ and $p27^{+/-}$ mice also show evidence of hyperplasia and dysplasia, which however, are generally less severe than that seen in *Nkx3.1* mutant mice. Compound mutant mice, particularly $p27^{-/-}$ $Nkx3.1^{-/-}$, $p27^{-/-}Nkx3.1^{+/-}$, and $p27^{+/-}Nkx3.1^{-/-}$ animals showed more extensive lesions with increasing nuclear crowding, nuclear elongation, and hyperchromasia (Figure 2).

We used a histopathological grading system in an attempt at objectively assessing the impact of loss of *Nkx3.1* and *p27* on prostatic pathology. Prostates are assigned scores from 0 to 4, with 0 representing normal looking epithelium and 4 representing the most severe lesions observed as detailed in the Materials and Methods section. As shown in Figure 3, complete loss of both alleles of *Nkx3.1* and *p27* leads to an increased incidence of PIN-like lesions (histopathological score 3 to 4). In this group ($p27^{-/-}Nkx3.1^{-/-}$), 100% of the animals have developed PIN-like lesions by 24 weeks of age. An increase in the incidence of PIN-like lesions was also observed in 36-week-old $p27^{-/-}Nkx3.1^{+/-}$ and $p27^{+/-}Nkx3.1^{-/-}$ mice (Figure 3). Histologically, the lesions in old $p27^{-/-}$ $Nkx3.1^{+/-}$ and $p27^{+/-}Nkx3.1^{-/-}$ animals were indistinguishable from those in $p27^{-/-}Nkx3.1^{-/-}$ mice. We were particularly interested in whether $p27^{+/-}Nkx3.1^{+/-}$ compound heterozygous mice will show evidence of cooperativity, because both *Nkx3.1* and *p27* are reported to show haploinsufficiency for tumor suppression.[4–6,12] Analysis of $p27^{+/-}Nkx3.1^{+/-}$ mice indicates no evidence for cooperativity in induction of PIN lesions in these animals (Figure 3) because the phenotype of $p27^{+/-}Nkx3.1^{+/-}$ mice was no worse than that of $p27^{+/-}Nkx3.1^{+/+}$ or $p27^{+/+}$ $Nkx3.1^{+/-}$ mice at all ages examined.

We further characterized the prostatic lesions in the mutant animals using morphometric analysis of Feulgen-stained nuclei. Using imaging software, nuclei were extracted from wild-type and $p27^{-/-}Nkx3.1^{-/-}$ double-knockout animals. Nuclei from mutant animals were significantly larger and had higher DNA content (Figure 4), confirming their dysplastic nature as judged by standard histopathological examination. To ascertain the degree to which our histopathological grading score correlates with morphometric analysis of Feulgen-stained nuclei, we used the image morphometric nuclear grading method of Bacus and colleagues.[15] The imaging software uses multiple parameters, including nuclear area, DNA mass, pleomorphism, entropy, circularity, elongation, coarseness, and angularity to calculate the image morphometric nuclear grade score. The image morphometric nuclear grade correlated well with the histopathogical grading score determined by examination of H&E-stained sections (Figure 4E).

Nkx3.1 and p27^{kip1} Cooperate to Suppress Proliferation in Prostatic Epithelium

We next examined the proliferative rates of prostate epithelial cells in the mutant mice using Ki67 staining. We have previously shown that Ki67 is a valid marker for, and correlates well with, DNA synthesis as measured by BrdU (bromodeoxyuridine) incorporation in mouse prostate epithelial cells.[7] Wild-type mouse prostate showed a low-Ki67-labeling index (Figure 5). Consistent with previous reports[5–7,10] loss of a single *Nkx3.1* or *p27* allele leads to a modest increase in Ki67 labeling (Figure 5). A further increase in Ki67-positive cells is seen when both alleles of either gene are lost ($p27^{-/-}Nkx3.1^{+/+}$ and $p27^{+/+}$ $Nkx3.1^{-/-}$). Double-knockout mice have a significant increase in Ki67 labeling indicative of a synergistic effect between the two molecules in suppressing the proliferation of prostatic epithelial cells. Consistent with the results of our histopathological examination, the proliferative rates in compound heterozygous, $p27^{+/-}Nkx3.1^{+/-}$ animals was the same as that in $p27^{+/-}Nkx3.1^{+/+}$ or $p27^{+/+}$ $Nkx3.1^{+/-}$ animals, indicating lack of cooperation when at least one allele of either gene is retained. Furthermore, the Ki67-labeling index of $p27^{-/-}Nkx3.1^{+/+}$ mice is not significantly different from that of $p27^{-/-}Nkx3.1^{+/-}$ animals. Nor did the Ki67-labeling index of $p27^{+/+}$ $Nkx3.1^{-/-}$ animals differ significantly from that of $p27^{+/-}$ $Nkx3.1^{-/-}$ mice. These results were confirmed by performing a similar quantitative analysis using a different marker associated with proliferation, the PCNA (Figure 5F). We thus conclude that *Nkx3.1* and *p27* regulate the proliferation of prostate epithelial cells through both haploinsufficient and nonhaploinsufficient pathways.

Altered Apoptosis in the Prostates of Nkx3.1 and p27^{kip1} Compound Mutant Mice

We used the TUNEL assay to examine apoptosis in the prostates of mutant mice. The apoptotic rate was low in the prostates of wild-type mice, and was not significantly altered in the prostates of $p27^{+/+}Nkx3.1^{+/-}$, $p27^{+/+}$ $Nkx3.1^{-/-}$, $p27^{+/-}Nkx3.1^{+/+}$, $p27^{+/-}Nkx3.1^{-/-}$, or $p27^{+/-}Nkx3.1^{+/-}$ animals (Figure 6). By contrast, the apoptotic rate was modestly but significantly increased in mutants lacking *p27* and retaining at least one allele of

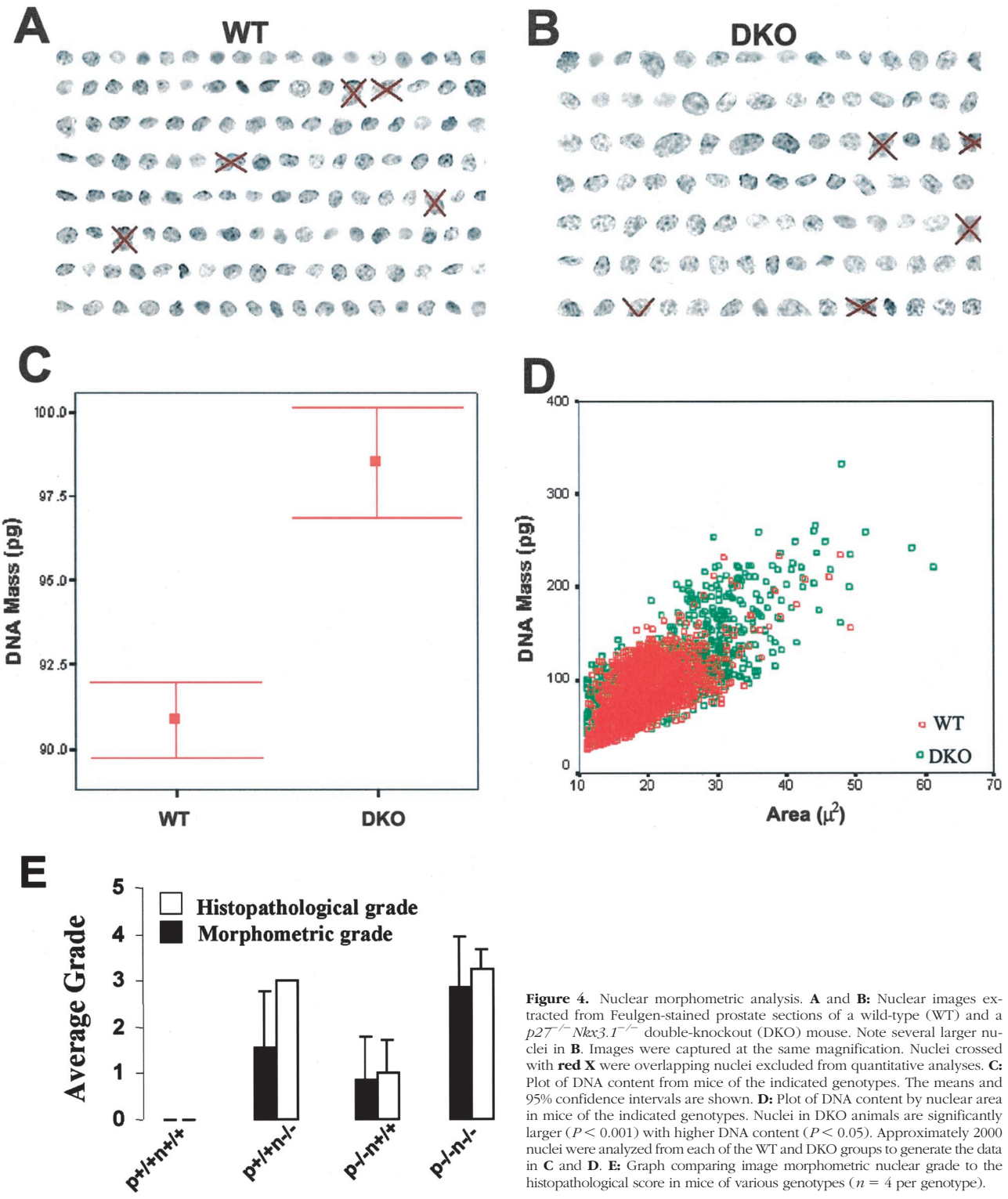

Figure 4. Nuclear morphometric analysis. **A** and **B:** Nuclear images extracted from Feulgen-stained prostate sections of a wild-type (WT) and a $p27^{-/-}Nkx3.1^{-/-}$ double-knockout (DKO) mouse. Note several larger nuclei in **B**. Images were captured at the same magnification. Nuclei crossed with **red X** were overlapping nuclei excluded from quantitative analyses. **C:** Plot of DNA content from mice of the indicated genotypes. The means and 95% confidence intervals are shown. **D:** Plot of DNA content by nuclear area in mice of the indicated genotypes. Nuclei in DKO animals are significantly larger ($P < 0.001$) with higher DNA content ($P < 0.05$). Approximately 2000 nuclei were analyzed from each of the WT and DKO groups to generate the data in **C** and **D**. **E:** Graph comparing image morphometric nuclear grade to the histopathological score in mice of various genotypes ($n = 4$ per genotype).

Nkx3.1 (ie, $p27^{-/-}Nkx3.1^{+/+}$ and $p27^{-/-}Nkx3.1^{+/-}$ mice, $P \leq 0.05$). However, this increase was not apparent in double-knockout mice ($p27^{-/-}Nkx3.1^{-/-}$), despite the striking increase in proliferation seen in these animals (Figure 5). These results suggest that lack of *p27* leads to an increase in apoptosis in the prostate, but this is suppressed in mice lacking *Nkx3.1*.

Loss of Nkx3.1 and $p27^{kip1}$ Protein Expression in PIN Lesions

Progression of prostatic epithelial hyperplasia to dysplasia is associated with loss of Nkx3.1 protein expression.[5,8] We therefore examined lesions from p27-deficient mice for evidence of loss of Nkx3.1 protein

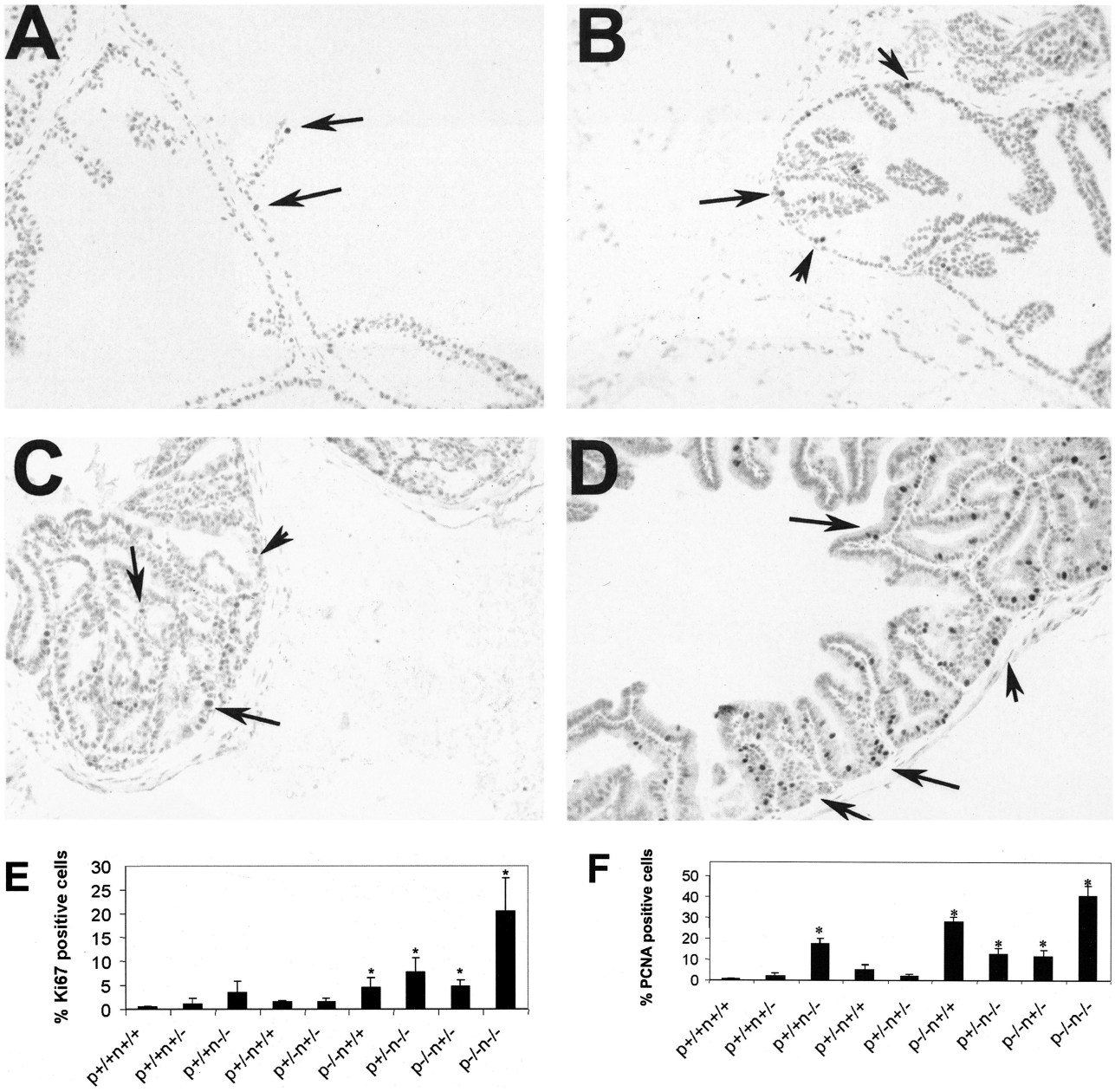

Figure 5. Nkx3.1 and p27 cooperate to suppress proliferation in prostatic epithelial cells. Ki67 immunohistochemistry marks proliferating prostate epithelial cells in the anterior lobes of 36-week-old mice: **A:** Wild-type; **B:** $p27^{-/-}$; **C**, $Nkx3.1^{-/-}$; **D**, $p27^{-/-}Nkx3.1^{-/-}$. **E:** Ki67-labeling index was determined in the anterior lobes of 36-week-old mice of various genotypes as indicated ($n = 3$ to 4 per genotype). *, $P < 0.05$ relative to wild-type. **F:** Graph showing PCNA-labeling index as determined in the anterior lobes of 36-week-old mice of the indicated genotypes ($n = 4$ per genotype). *, $P < 0.05$ relative to wild-type.

expression. We found focal areas of loss of Nkx3.1 expression in lesions from $p27^{-/-}Nkx3.1^{+/-}$ and $p27^{-/-}Nkx3.1^{+/+}$ mice (Figure 7). To determine whether $p27^{kip1}$ expression is lost in Nkx3.1 mutant prostates, we examined the lesions of $p27^{+/-}Nkx3.1^{-/-}$ and $p27^{+/-}Nkx3.1^{+/-}$ animals. In contrast to wild-type prostates, which show a more uniform expression of $p27^{kip1}$, the mutant prostates exhibit a more heterogeneous expression, with areas of focal loss of expression (Figure 7). Thus cells exist in foci of dysplastic cells in compound mutant mice where protein expression from the wild-type allele is silenced, effectively rendering the involved cells null for the protein in question ($p27^{kip1}$ or Nkx3.1). In sum, these results support the notion that loss of Nkx3.1 and $p27^{kip1}$ protein expression, which are observed in PIN lesions in human prostate carcinoma may have a role in promoting prostate tumor initiation.

Discussion

Details of the processes that regulate prostate cancer initiation remain poorly understood. Current evidence points to PIN as a precursor lesion for prostate cancer, yet the genetic and molecular changes required for benign prostate epithelial cells to develop into PIN are not

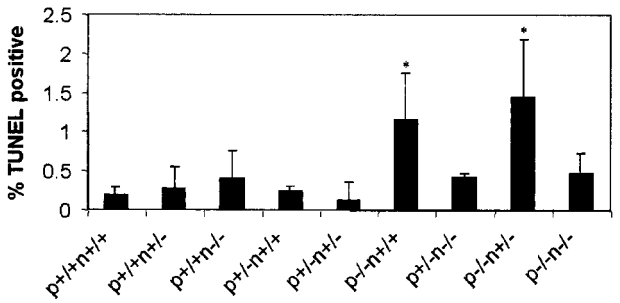

Figure 6. Analysis of apoptosis in prostates of compound mutant mice. The percentages of TUNEL-positive cells were determined in the anterior prostates of 36-week-old mice of various genotypes as indicated ($n = 3$ to 4 per genotype). The means and standard deviations are shown. *, $P < 0.05$ relative to wild-type.

fully understood. Recently, the prostate-specific homeodomain transcription factor NKX3.1 has emerged as a candidate tumor suppressor gene whose loss is associated with prostate tumor initiation.[19] Re-expression of NKX3.1 in human prostate carcinoma cell lines negative for NKX3.1 results in suppression of growth and tumorigenicity, and deletion of Nkx3.1 in mice results in prostatic epithelial hyperplasia and PIN.[4–6]

An interesting aspect of Nkx3.1 regulation of prostatic proliferation involves the phenomenon of haploinsufficiency. A benign cell has to overcome many obstacles *in situ* to develop into a fully malignant clone, and the chances of accumulating multiple genetic lesions concurrently in a normal cell are extremely low.[20] Haploinsufficiency has been proposed as a mechanism by which the efficiency of tumorigenesis can be increased. Loss of a single allele of an haploinsufficient tumor suppressor is sufficient to confer some growth advantage on the mutated cell, thereby expanding the target population in which subsequent mutations can occur. Both *p27* and *Nkx3.1* have been shown to demonstrate haploinsufficiency. The mechanistic basis for haploinsufficiency in Nkx3.1 mutant mice has begun to be explored. Loss of a single *Nkx3.1* allele was found to extend the proliferative phase of luminal epithelial cells of the prostate.[7] This is associated with the loss of a subset of exquisitely dosage-sensitive *Nkx3.1* target genes. Loss of the second *Nkx3.1* allele is associated with dysregulation of another subset of target genes. The results presented here indicate that both *p27* and *Nkx3.1* regulate prostate epithelial proliferation and development of preneoplastic lesions through both haploinsufficient and nonhaploinsufficient pathways. Partial loss of *p27* and *Nkx3.1* (ie, compound heterozygosity) did not lead to a significant additive effect on proliferation or development of dysplasia. By contrast, complete loss of both genes resulted in a synergistic increase in proliferation, which is reflected in the increased efficiency with which PIN-like lesions develop.

A notable feature of our studies is the finding that complete loss of *p27* expression has an effect on the rate of prostatic epithelial cell apoptosis. The apoptotic rate was increased in $p27^{-/-}$ prostates, and this may explain why the prostatic lesions in these animals are limited. Although p27 is primarily involved in regulating the cell cycle, an increase in apoptosis has previously been observed in some tissues of the p27-deficient mouse, for example the retina.[21] Conversely, in *Rb*-deficient cells, *p27* appears to function as a proapoptotic tumor suppressor.[22] Interestingly, our results imply that loss of *Nkx3.1* suppresses apoptosis in *p27*-deficient prostates, because the apoptotic rate in $p27^{-/-}Nkx3.1^{-/-}$ prostates was lower that that in the prostates of $p27^{-/-}$ animals. The precise mechanisms for this phenomenon are presently unclear. Nevertheless, these results suggest that foci of dysplastic cells in PIN lesions that lose both p27 and Nk3.1 protein expression may have an advantage over neighboring cells in both increased proliferation and decreased apoptosis.

Our observations indicating that lesions in p27/Nkx3.1 compound mutant mice do not rapidly progress to invasive prostate cancer is consistent with reported observa-

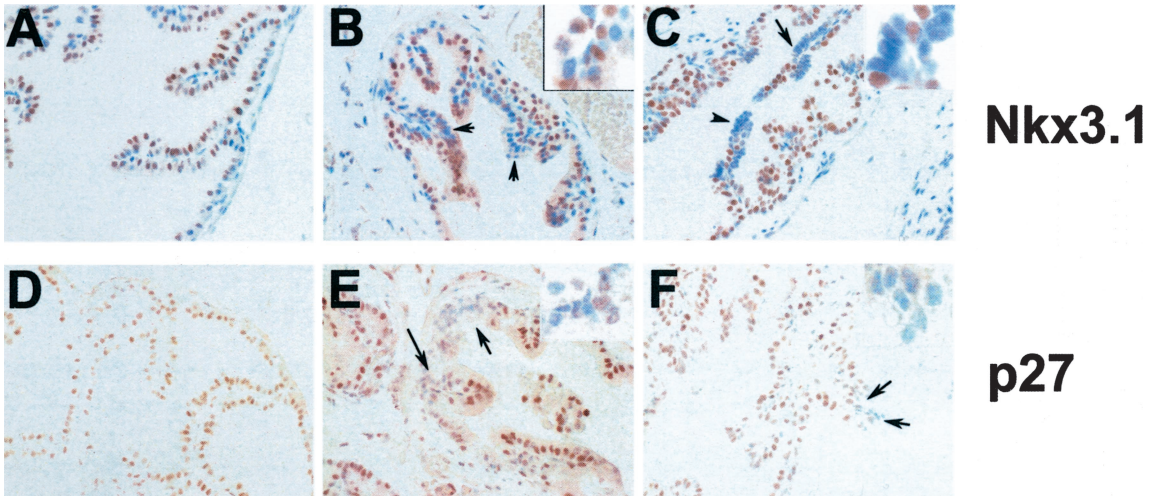

Figure 7. Focal loss of Nkx3.1 and p27 protein expression in prostate lesions from compound mutant mice. **A–C:** Immunohistochemical analysis of Nkx3.1 protein expression in the anterior prostates of 36-week-old mice. **D–F:** p27 immunohistochemistry in the anterior prostates of 36-week-old mice. **A** and **D:** Wild-type mice show relatively uniform nuclear expression of Nkx3.1 and p27, respectively, in luminal epithelial cells. $p27^{-/-}Nkx3.1^{+/-}$ (**B**) and $p27^{-/-}Nkx3.1^{+/+}$ (**C**) prostates show focal areas of loss of Nkx3.1 staining (**arrows** and **inset**s). $p27^{+/-}Nkx3.1^{-/-}$ (**E**) and $p27^{+/-}Nkx3.1^{+/-}$ (**F**) prostates show areas of focal loss of p27 expression (**arrows** and **insets**). Original magnifications: ×40; ×100 (**insets**).

tions from several genetically engineered mice not based on SV40 T antigen.[14,23] This has been attributed to the low-proliferative rate of the mouse prostate and the need to disrupt multiple tumorigenic pathways for progression to advanced invasive cancer to occur. Nevertheless, although our studies focus on the roles of *Nkx3.1* and *p27* in the early stages of prostate tumorigenesis, they do not rule out additional effects of these genes in established tumors. This possibility needs to be entertained because loss of p27 and Nkx3.1 have each been shown to correlate with patient prognosis in human tumors[3,24] implying that tumors that developed through selection for loss of expression of these proteins are more aggressive. Finally, our studies indicate that p27 and Nkx3.1 play distinct roles in prostate tumor initiation, and that these molecules function by affecting both haploinsufficient and nonhaploinsufficient pathways.

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